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Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses

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Summary

We used the cytokinin-responsive *Arabidopsis response regulator* (*ARR*)*5* gene promoter fused to a β-glucuronidase (*GUS*) reporter gene, and cytokinin oxidase (*CKX*) genes from *Arabidopsis thaliana* (*AtCKX3*) and maize (*ZmCKX1*) to investigate the roles of cytokinins in lateral root formation and symbiosis in *Lotus japonicus*. *ARR5* expression was undetectable in the dividing initial cells at early stages of lateral root formation, but later we observed high expression in the base of the lateral root primordium. The root tip continues to express *ARR5* during subsequent development of the lateral root. These results suggest a dynamic role for cytokinin in lateral root development. We observed *ARR5* expression in curled/deformed root hairs, and also in nodule primordia in response to Rhizobial inoculation. This expression declined once the nodule emerged from the parent root. Root penetration and migration of root-knot nematode (RKN) second-stage larvae (L2) did not elevate *ARR5* expression, but a high level of expression was induced when L2 reached the differentiating vascular bundle and during early stages of the nematode–plant interaction. *ARR5* expression was specifically absent in mature giant cells (GCs), although dividing cells around the GCs continued to express this reporter. The same pattern was observed using a green fluorescent protein (*GFP*) reporter driven by the *ARR5* promoter in tomato. Overexpression of *CKX* genes rendered the transgenic hairy roots resistant to exogenous application of the cytokinin [N^6-(Δ^2 isopentenyl) adenine riboside] (iPR). *CKX* roots have significantly more lateral roots, but fewer nodules and nematode-induced root galls per plant, than control hairy roots.

Keywords: cytokinins, lateral root, symbiosis, cytokinin oxidase, *ARR*, *Lotus japonicus*.

Introduction

For most vascular plants, root growth occurs through the formation of lateral roots from meristems formed *de novo* in differentiated root tissues (Malamy and Benfey, 1997a,b). Certain plant-associated microorganisms also initiate new root meristems, from which the various organs central to the ensuant symbiotic associations are derived. The best understood of these organs are nitrogen-fixing nodules, which form on legume roots in interaction with Rhizobia (Schultze and Kondorosi, 1998). Nematode–plant symbioses are parasitic, and lead to yield loss in the world's major food and fiber crops exceeding 12% annually (Koenning *et al.*, 1999; Sasser and Freckman, 1987). The best studied group are the root-knot nematodes (RKNs: *Meloidogyne* spp.), and although the feeding structures induced by RKN have not typically been described as plant organs, the cells at their core (giant cells (GCs)) share many features with meristems (Bird and Kaloshian, 2003).

Features of lateral root and nodule development in legumes have been compared (Hirsch, 1992; Koltai *et al.*, 2001; Libbenga and Bogers, 1974; Nutman, 1948, 1949), and plant hormone cytokinins have been implicated in the formation of both organs (Hirsch *et al.*, 1995; Schmülling, 2002). Transgenic tobacco plants expressing the cytokinin biosynthesis gene *isopentenyl transferase* exhibit reduced

root growth (Hewelt *et al.*, 1994). Similarly, exogenous application of the cytokinin 6-benzylaminopurine (BA) to *Lactuca sativa* reduced the number of lateral roots by 50% (Zhang and Hasenstein, 1999). Werner *et al.* (2001) reported an increase in the number of lateral roots in transgenic tobacco plants overexpressing *Arabidopsis* cytokinin oxidase (*CKX*) genes. Collectively, the experimental evidence suggests that cytokinins have inhibitory effect on lateral root formation. In contrast, exogenous application of low levels of BA (1 μM) to pea roots (Lorteau *et al.*, 2001) leads to an increase in Rhizobial nodulation (although at higher concentrations, the nodule number decreased), and molecular data are consistent with this. The early nodulation gene *ENOD40* from *Medicago sativa* is induced after exogenous cytokinin application (Fang and Hirsch, 1998; Hirsch *et al.*, 1995), and Rhizobial and cytokinin induction of white clover *ENOD40* expression is both spatially and temporally similar in nodule progenitor cells (Mathesius *et al.*, 2000). Bauer *et al.* (1996) reported a similar pattern of alfalfa cortical cell division (marking the onset of nodule initiation), following root treatment with either purified Nod factor or cytokinin. The cytokinin response required both active photosynthesis and a limiting nitrogen supply, thus recapitulating the response to Nod factor. RKN induces galls and GCs on essentially all vascular plants (Sasser, 1980), and biochemical measurements revealed increased cytokinin levels in RKN-infected roots (Bird and Loveys, 1980). Intriguingly, the nematodes themselves produce biologically active cytokinin (Bird and Loveys, 1980; de Meutter *et al.*, 2003), although whether this plays any role in the host–parasite interaction is unclear.

The discovery of cytokinin response genes in *Arabidopsis* (Brandstatter and Kieber, 1998) permits construction of promoter–reporter gene fusions to map cytokinin responses during lateral root formation, and infection by Rhizobia and RKN in transgenic plants. Expression of this reporter results from increased endogenous cytokinin levels and/or increased sensitivity to cytokinin. Similarly, the cloning of *CKX* genes (Morris *et al.*, 1999; Werner *et al.*, 2001) has made it possible to construct transgenic plants in which cytokinin levels can be lowered. We have used these tools to map and modulate cytokinin levels during normal plant development, and during the symbiotic interactions with nematodes and Rhizobia. Although *Arabidopsis* is a RKN host (albeit a poor one), it fails to nodulate. In contrast, the model legume *Lotus japonicus* supports Rhizobial nodulation and robust RKN gall formation (Lohar and Bird, 2003). We used the promoter of a cytokinin primary-response gene *Arabidopsis response regulator* (*ARR*)*5* (D'Agostino *et al.*, 2000) fused to a β-glucuronidase (*GUS*) reporter gene to map the status of cytokinin responses in a given tissue in transgenic *Lotus* hairy roots. The expression of *ARR5* in response to RKN was further validated by *in situ* detection of transcripts in transgenic tomato plants using a green fluorescent protein (*GFP*) reporter. Further, we made cytokinin-resistant transgenic *L. japonicus* hairy roots using *CKX* genes from *Arabidopsis thaliana* (*AtCKX3*) and maize (*ZmCKX1*), and showed that these roots had significantly increased lateral roots but reduced root nodules and RKN galls compared to control hairy roots. Collectively, our results support a model in which cytokinins play opposite roles in lateral root formation, compared to RKN and Rhizobial symbioses.

Results

Stereotypical induction of β-glucuronidase expression in L. japonicus *roots from the* ARR5 *promoter by endogenous cytokinins*

As a surrogate to spatially and temporally map endogenous cytokinin levels, we constructed *L. japonicus* hairy roots transgenic for the cytokinin-responsive *ARR5* promoter driving a *GUS* reporter gene. *ARR5* has been comprehensively authenticated as exhibiting a primary response to cytokinin. Its induction is cytokinin-specific (Brandstatter and Kieber, 1998), with elevated transcript levels reaching steady state within 10 min of application of exogenous cytokinin (as BA), and also is resistant to inhibitors of protein synthesis. In the absence of exogenous BA application (the basal expression pattern), GUS staining is seen in the primary and lateral root tips of *Arabidopsis*, presumed to be the meristems (D'Agostino *et al.*, 2000). These workers also observed patchy intermittent vascular staining in mid-portions of the root.

Based on observation of more than 50 independent transgenic roots, we found the pattern of endogenous GUS staining driven by the *ARR5* promoter in *L. japonicus* transgenic hairy roots to be essentially identical to that described for *A. thaliana* (Figure 1). GUS activity was observed in root tips (Figure 1a,b) and in the vascular cylinder in the root (Figure 1a,c,d). In the root tip, the characteristic staining was mainly restricted to the meristem area in and around the quiescent center and in some cases, columella root cap cells (Figure 1b). Very occasionally, the whole root tip, including the root cap, was stained (data not presented). As is the case in *Arabidopsis* (D'Agostino *et al.*, 2000), GUS staining in the vascular bundle was in intermittent patches (Figure 1a). The absence of staining above the root tip was observed as a characteristic in the transgenic hairy roots containing the cytokinin-inducible GUS gene. A transverse section of the GUS-stained root indicated that the staining in the vascular bundle was probably in the pericycle (Figure 1d). In some roots, a few cortical cells and some root hairs occasionally were stained. Addition of exogenous cytokinin as BA at 5 (Figure 1e) and 10 μM (Figure 1f) resulted in

strong induction of the *ARR5* promoter, seen as strong GUS induction compared to roots without BA (Figure 1a). Collectively, these results confirmed that the *ARR5* promoter functions in *L. japonicus* in a manner similar to that in its cognate plant (*Arabidopsis*), thus validating its use in this study. It is important to note that all cells of the root respond to cytokinin by upregulation of *ARR5*, implying that all cells have the capacity to respond to this hormone and that *ARR5* expression provides an effective surrogate for the state of cytokinin signaling in a given cell.

Expression of ARR5 *during lateral root formation*

Although the *ARR5* promoter is active in the primary root meristem of *Arabidopsis* (D'Agostino *et al.*, 2000) and *Lotus* (Figure 1b), lateral roots originated from meristems, which were distinct and distant from the primary meristem, and conceivably might arise from regions of the mature root in which the *ARR5* promoter was active (stained in Figure 1a) or silent (not stained). Using the *ARR5::GUS* reporter as a surrogate to establish the pattern of cytokinin responsiveness during initiation of lateral roots, we examined lateral root formation both in parts of the root exhibiting a general cytokinin response in the vascular bundle, and in portions of the root with little or no vascular staining (Figure 2a,b). Strikingly, in every instance where lateral root was initiated in a *ARR5* expression zone (blue), a specific absence of expression was observed in those pericycle cells undergoing division to initiate the lateral root primordium (Figure 2a). This stage of lateral root formation corresponds to stage III or IV in *Arabidopsis* (Malamy and Benfey, 1997a). This presumably reflects repression of the cytokinin response, either via decreased levels or sensitivity, as spatially equivalent pericycle cells not undergoing organogenesis clearly exhibit GUS staining (Figure 1d). Conversely, there was no expression of *ARR5* in the dividing cells of the early lateral root primordium in the portion of the root without a vascular cytokinin response (Figure 2b). Thus, initiation of lateral roots is associated with a localized repression of any pre-existing cytokinin response. The developing lateral root primordium continued not to express *ARR5* during the few subsequent cell divisions (Figure 2c,d), and expression was not restored until the lateral root primordium assumed the initial cone shape of a root tip (stage VIb). At this point, *ARR5* expression was observed at the base of the cone, originating in the parent root vascular bundle (Figure 2e), likely reflecting formation of an organized meristem in the developing lateral root. Restriction of *ARR5* expression to the new meristem persists as the lateral root erupts from the parent root (Figure 2f–i). Together, these results indicate that cytokinin sensitivity and/or levels are spatially and temporally regulated during lateral root formation.

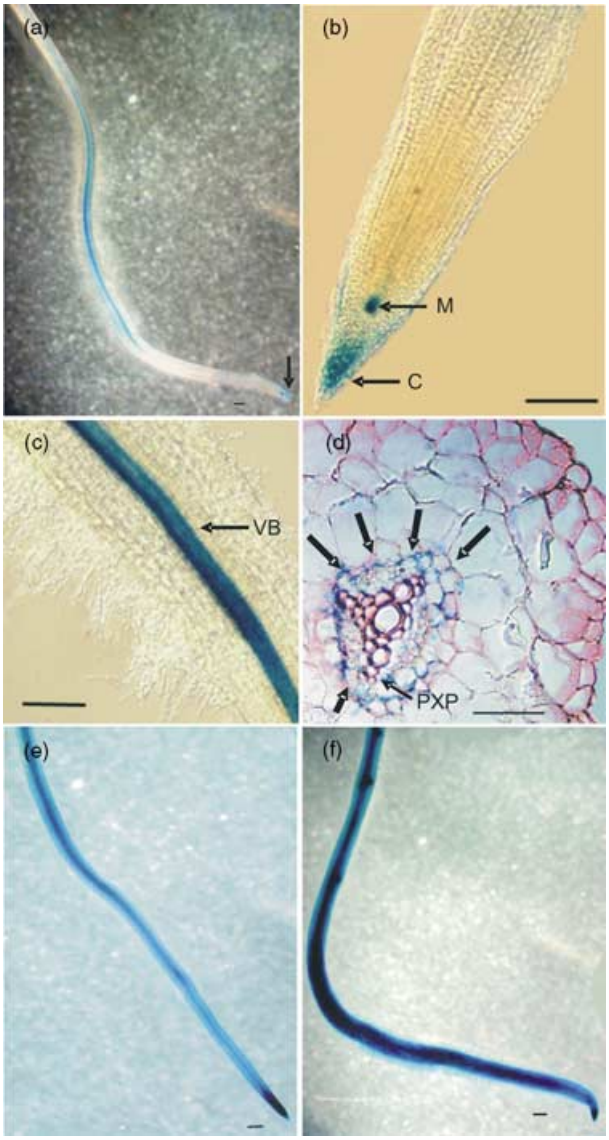

Figure 1. *Lotus japonicus* roots transgenic for *ARR5* promoter::*GUS* fusion exhibit a stereotypical response to endogenous and exogenous cytokinins. Blue staining indicates GUS expression, and hence a cytokinin response.
(a) Root with a cytokinin response in the root vascular bundle and root tip.
(b) Cytokinin response in the root tip, showing staining in the meristematic region (M), and the columella root cap (C); the columella root cap is not stained in all root tips.
(c) Cytokinin response in the root vascular bundle (VB) of the root.
(d) Transverse section through the root as in (c) showing GUS induction in pericycle cells (dark arrows) in the vascular bundle, counter-stained with Safranin O; PXP, protoxylem pole.
(e) GUS induction following exogenous root application of 5 μM BA.
(f) GUS induction following exogenous root application of 10 μM BA.
Bars in (a–c) and (e,f) = 100 μm; bar in (d) = 50 μm.

Expression of ARR5 *during nodule formation*

To map *ARR5* expression during nodulation, we inoculated *L. japonicus* hairy roots transgenic for the *ARR5::GUS* construct with *Mesorhizobium loti* and visualized GUS

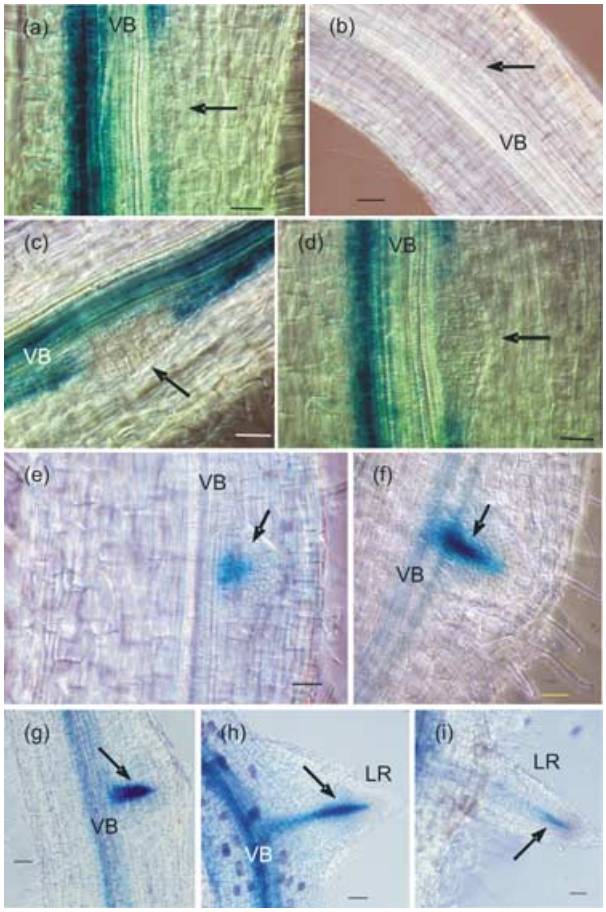

Figure 2. *Arabidopsis response regulator 5* expression during lateral root formation in *L. japonicus* roots.
(a) Repression of *ARR* expression in the lateral root primordium (stage III or IV) in the part of the root exhibiting a general, vascular expression. Note the strong expression in the outer vascular bundle opposite and on either side of the lateral root primordium.
(b) *ARR5* expression is not induced in the lateral root primordium (stage III or IV) in the part of the root not exhibiting general *ARR5* expression in the vascular bundle.
(c, d) Developing lateral root primordia (stages Va and Vb, respectively) with no *ARR5* induction.
(e–i) Induction of *ARR5* expression (arrows) in the lateral root meristem during lateral root development. (e) stage VIa; (f) stage VIb; (g) stage VII; (h,i) emergence. VB, vascular bundle; LR, lateral root.
Blue staining indicates GUS expression, and hence *ARR5* expression. Results are typical of observations from more than 25 independent transgenic roots. *ARR5* expression is absent during the early stages (stages III–Vb) of lateral root formation (a–d). Arrows indicate the nascent lateral root primordium, and its development into a meristem. Bars in (a–e) = 25 μm; bars in (f–i) = 50 μm.

expression by β-glucuronic acid staining. Strikingly, the earliest induction of *ARR5* we observed on Rhizobia-inoculated roots was in the curled/deformed root hairs 48 h post-inoculation (Figure 3a). Mock-inoculated roots failed to exhibit root hair deformation, and except for an occasional, very weak response in some root hairs, did not show *ARR5*-induced GUS expression (Figure 3b). As a further control, we inoculated the transgenic hairy roots with a strain of *M. loti* carrying a *Tn5* insertion in *nodC*, which abolishes the ability to make Nod factor, and obtained the same result as with mock-inoculation, i.e. no root hair curling and cytokinin response (Figure 3c).

We continued to monitor roots inoculated with Rhizobia, and observed induction of the *ARR5* promoter in the dividing cortical cells (Figure 3d), and presumed the cytokinin-responsive cortical cells to be the initials of nodule primordia. The extent of *ARR5* activity increased *pari passu* with the expansion of the nodule primordium (Figure 3e–g). However, by the stage of development when the nodule was about to emerge from the parent root, the only cytokinin response evident was a weak signal confined to the periphery of the nodule (Figure 3h). In an emerged young nodule, GUS activity was observed solely in some outer parts of the nodule (Figure 3i), and became undetectable in mature nitrogen-fixing nodules (Figure 3j).

Expression of ARR5 *during infection by root-knot nematode*

We monitored cytokinin-inducible GUS expression in the *L. japonicus ARR5* transgenic hairy roots following inoculation with either *Meloidogyne incognita* or *M. hapla* second-stage larvae (L2). These species have a stereotypical pattern of root penetration in the zone of elongation, followed by intercellular migration to the meristem and then into the developing stele in *Lotus* (Lohar and Bird, 2003). *ARR5* expression in the area of worm entry and migration through the cortex was not apparent. However, once the larvae moved into the differentiating vascular bundle, strong induction of *ARR5* expression was readily apparent (Figure 4a), extending from the meristem (Figure 1b) into the developing vasculature.

A characteristic feature of the RKN–plant interaction is the induction of GC as the core of a root gall. Based on GUS staining, we found that no matter whether a feeding site was initiated in a region of the root either displaying strong vascular *ARR5* expression or not (Figure 4b,c), the *ARR5* promoter became active in the nascent gall (Figure 4b,c). This high level of *ARR5* expression remained during later stages of development of the *M. incognita*-induced galls (Figure 4d). However, careful examination suggested that cells at the center of mature galls lack GUS staining (Figure 4d). The anatomy of an *M. incognita*-induced gall in which several GCs are surrounded by layers of rapidly dividing small cells, which themselves are surrounded by several layers of expanded cortical cells, is shown in Figure 4(e). A similar transverse section through section of a X-gal-stained gall (Figure 4f) confirmed that *ARR5* expression was primarily restricted to the rapidly dividing small cells around the GC; whereas the GCs themselves did not express the reporter. Essentially, the same results were obtained when transgenic *L. japonicus ARR5::GUS* plants

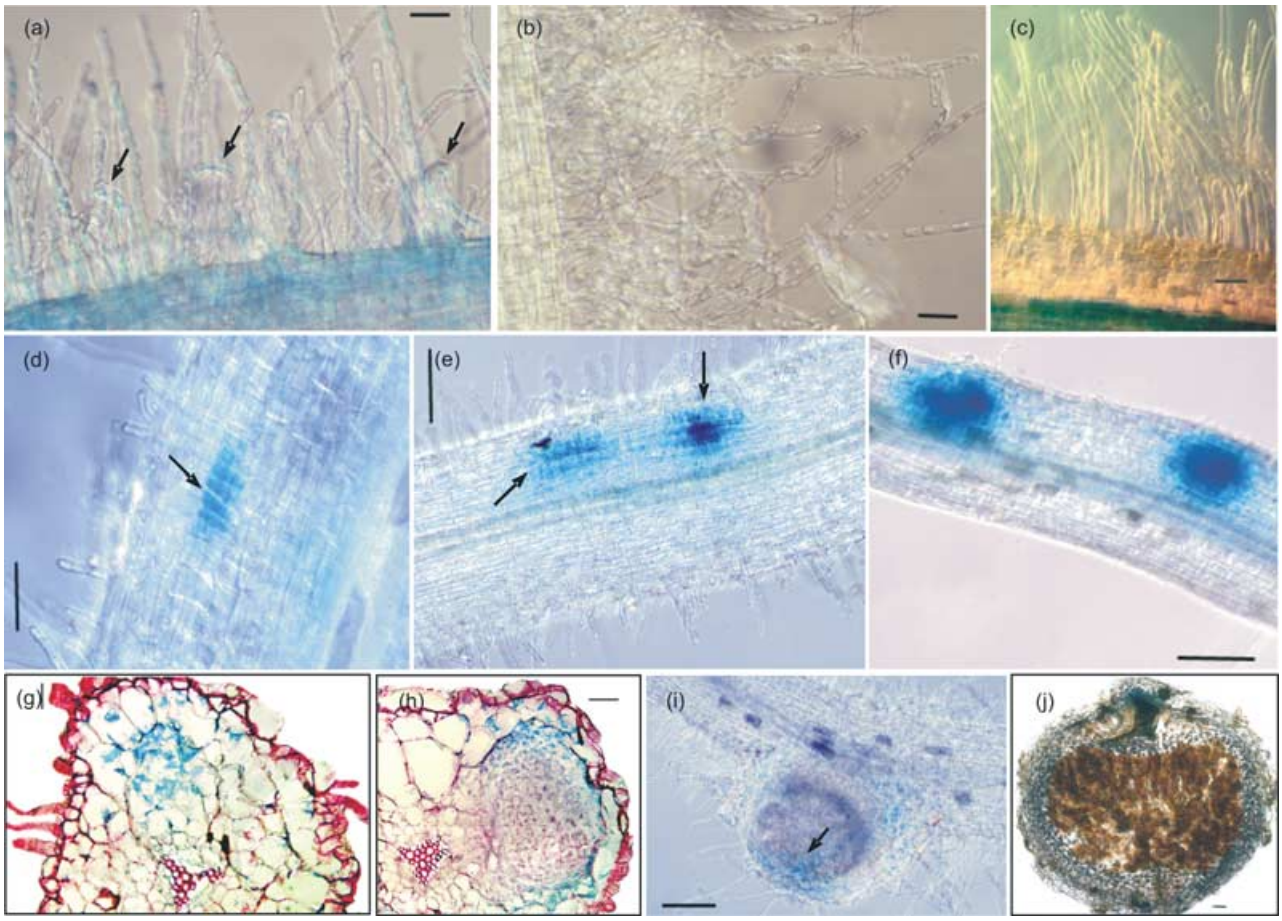

Figure 3. Expression of *ARR5* in *L. japonicus* during symbiosis with *M. loti*.
Host plants transgenic for the *ARR5* promoter::*GUS* fusion construct, which reports a cytokinin response as blue staining, were inoculated either with a wild-type *M. loti* strain (NZP 2235) (a,d–j), an *M. loti* nodulation defective mutant (*NodC::Tn5*) (c), or were mock-inoculated (b). Results are typical of observations from more than 25 independent transgenic roots. The wild-type Rhizobial strain elicits root hair curling (arrows; a) and induction of *ARR5* expression in root hairs and in the dividing cells of the nodule primordium (arrow; d). *ARR5* expression (arrows) is evident in later developing nodules especially in the dividing cortical cells (e). A late stage of nodule formation shows strong *ARR5* expression in the primordium (f) and in dividing cortical cells (g). No *ARR5* expression is evident in the nodule in a cross section of a root with a nodule about to emerge from the parent root (h), although some expression is present in the periphery of the nodule. In a fully emerged nodule, *ARR5* expression is obvious only in some areas in the periphery (arrow; i) of the nodule. A hand-section of a mature nitrogen-fixing nodule revealed no *ARR5* expression in the nodule (j), but expression in the parent root vascular bundle is evident. Sections were counter-stained with Safranin O (g,h). Bars in (a–f,h,i) = 50 μm; bar in (g) = 25 μm; bar in (j) = 100 μm.

were inoculated with *M. hapla* (not shown), although fewer layers of small dividing cells were observed around these GCs, presumably leading to the smaller galls typically associated with *M. hapla* infection.

To independently confirm these observations, we infected tomato plants transgenic for the *ARR5* promoter driving a *GFP* reporter gene with *M. incognita* L2. Precisely as was the case in *Lotus*, mature nematode-induced galls exhibited a cytokinin response (Figure 5a,b). To establish the pattern with cellular resolution, we localized the *GFP* transcripts *in situ* (Koltai and Bird, 2000). Compared to an *18S rRNA* control (Figure 5c) in which strong GC staining was evident, *GFP* mRNA was not detected in the GC (Figure 5d), although weak staining was detected in cells of the surrounding gall. No staining was apparent in a no-primer control (not shown). The chosen primers were able to amplify the expected product in mRNA from root tips (Figure 5e).

Collectively, results from tomato and *Lotus* indicate that the *ARR5* cytokinin-responsive promoter is first activated following RKN entry into the vascular stele, but prior to the initiation of feeding sites by the L2. The galls induced by RKN also exhibit *ARR5* expression, but at established feeding sites, the stereotypic GCs do not show *ARR5* activity.

Transgenic CKX *hairy roots have increased lateral roots but reduced root nodules and RKN galls*

Transgenic expression of *CKX* genes provides a means to reduce cytokinin levels *in planta*, and so we constructed *L. japonicus* hairy roots carrying either *CKX3* from *Arabidopsis* (*AtCKX3*) or *CKX1* from maize (*ZmCKX1*), both

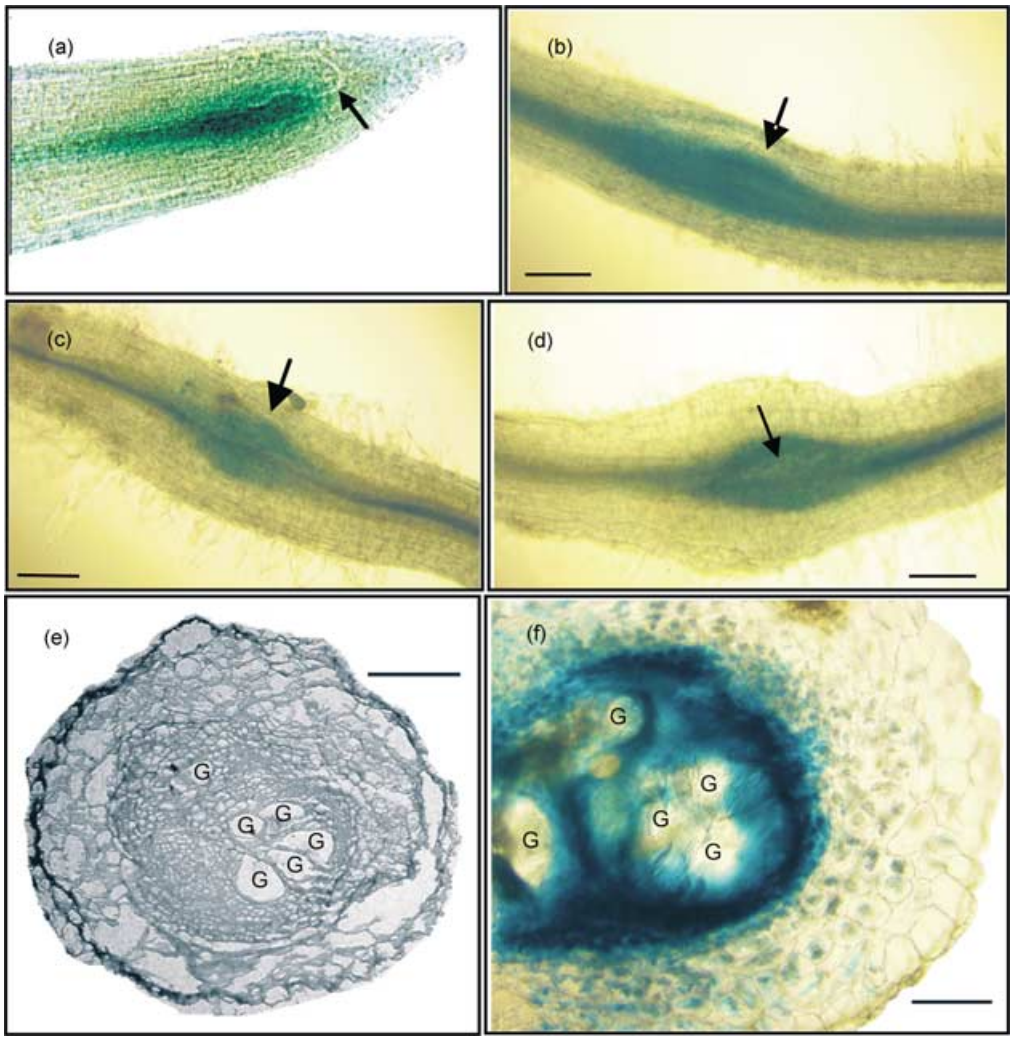

Figure 4. Cytokinin-inducible GUS expression following *M. incognita* invasion of *L. japonicus* transgenic hairy roots.
(a) *Meloidogyne incognita* L2 (arrow) entering the root tip and migrating towards the root vascular cylinder. *ARR5* expression is induced when larvae reaches the differentiating vascular bundle above the root apical meristem. For *ARR5* expression in an uninoculated root, see Figure 1(b).
(b) *ARR5* expression during feeding site induction (arrow) by *M. incognita* in the part of the root with vascular staining.
(c) *ARR5* expression during feeding site induction (arrow) by *M. incognita* in the part of the root without vascular staining.
(d) *ARR5* expression in an *M. incognita*-induced young gall; note the lack of GUS staining in the center of the gall (arrow).
(e) Unstained, transverse section through a mature gall to show morphology. GCs (G) are surrounded by proliferating small cells. Outside the cell proliferation areas are enlarged cortical cells.
(f) A transverse section through a mature gall stained with X-gal. GCs (G) are surrounded by dividing cells with high-level expression of *ARR5*. The outer enlarged cortical cells do not express detectable levels of the *ARR5* reporter.
Roots were inoculated with freshly isolated eggs, and were sampled starting a week after inoculation for X-gal staining. Bars in (a–d) = 100 μm; bars in (e,f) = 200 μm.

driven by the *CaMV 35S* promoter. We also generated vector-only transgenic hairy roots as controls. Because the constructs lack a visible marker, we performed PCR on total DNA isolated from independent hairy roots to establish the presence of the transgenes. This confirmed that most of the hairy roots were positive for the transgenes (Figure 6a,b), with only one *ZmCKX1* hairy root failing to produce an expected product (Figure 6b). In contrast, all 10 independent hairy roots generated using vector alone were found negative by the lack of expected PCR products (Figure 6a,b). Transgenic lines thus validated were grown as composite plants (a transgenic hairy root and wild-type shoot) in the presence of the cytokinin [N^6-(Δ^2 isopentenyl) adenine riboside] (iPR) to test for insensitivity to exogenous cytokinins. At all iPR concentrations tested, hairy roots transgenic for *AtCKX3* or *ZmCKX1* were significantly less sensitive to exogenous iPR application than control hairy roots, as scored by relative root growth (Figure 6c). Root growth of control hairy roots and *CKX* roots was significantly equivalent in the absence of exogenous iPR.

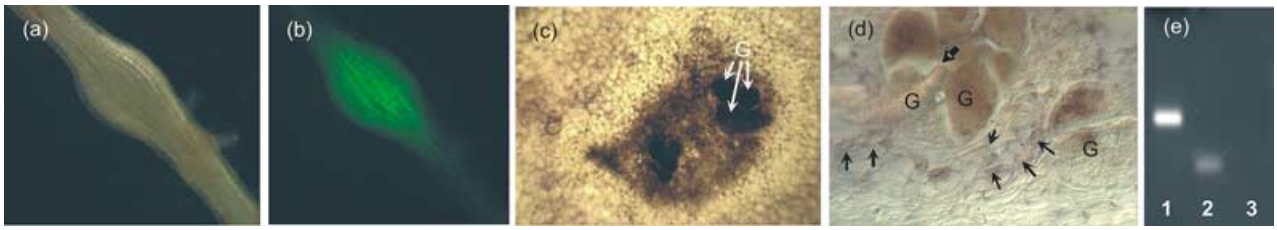

Figure 5. Cytokinin-inducible *GFP* expression following *M. incognita* invasion of transgenic tomato.
(a) Bright-field image of gall 2 weeks after infection.
(b) Fluorescent image of (a). *ARR5* expression is evident as green fluorescence.
(c) *In situ* localization of *18S rRNA* transcripts (dark staining) in a transverse section through a 3-week-old gall. All cells exhibit staining, but the GCs (G) are especially strongly stained.
(d) *In situ* localization of *GFP* transcripts (blue-brown staining) in a transverse section through a 3-week-old gall. GCs (G) show no staining; the beige color seen is a typical background for these cytoplasmically dense cells (Koltai *et al.*, 2001). Weak staining (thin arrows) is evident in some cells of the developing gall surrounding the GCs. The head of the nematode is apparent (thick arrow), but the body is in a different focal plane.
(e) Validation of *GFP* primer pair used for (c) by RT-PCR amplification of root RNA, using primers specific for *18S rRNA* (lane 1: 350 bp) and *GFP* (lane 2: 124 bp). The primer for reverse transcription was omitted from the reaction shown in lane 3.

However, root growth of control hairy roots in the presence of iPR was significantly less than in the absence of iPR. In contrast, *CKX* hairy root growth was not significantly inhibited by exogenous iPR application. These results indicated that the *CKX* hairy roots were less sensitive to exogenous cytokinins than the control hairy roots.

Transgenic *CKX* roots had significantly more lateral roots than the controls (Figure 6d). There was no significant difference for lateral root number between roots overexpressing *AtCKX3* or *ZmCKX1*. To quantify nodulation in *CKX* plants, *M. loti* NZP2235 was used as inoculum on transgenic hairy roots, and the mean number of nodules per plant in the control and *CKX* transgenic roots was determined (Figure 6d). Both *AtCKX3* and *ZmCKX1* transgenic hairy roots had significantly reduced nodule number per plant (Figures 6d and 7a). However, there was no significant difference in nodule number per plant between *AtCKX3* and *ZmCKX1* roots. Some of the *CKX* transgenic roots entirely failed to nodulate. We interpret the reduced nodule number in *CKX* transgenic hairy roots to be reflective of reduced sensitivity to cytokinins in these plants.

We infected transgenic hairy roots with freshly isolated *M. incognita* eggs to see if cytokinin resistance had any effect on the root–RKN interaction, as determined by gall number per root 3 weeks after inoculation. Hairy roots with *AtCKX3* and *ZmCKX1* as transgenes had less than 50% galls compared to control hairy roots, and the difference was statistically significant (Figure 6d). Thus, cytokinin-resistant hairy roots were less susceptible to RKN than the normal hairy roots. We also observed that the galls induced on cytokinin-resistant hairy roots were smaller in size than those induced on control roots (Figure 7b).

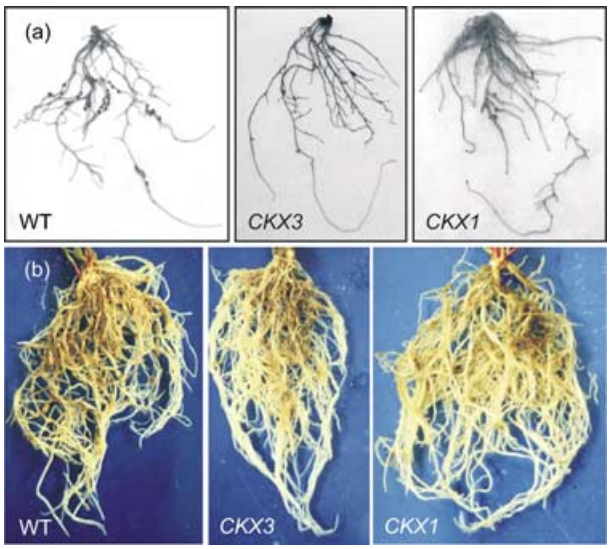

Figure 7. *Lotus japonicus* control (WT) or hairy roots transgenic for *AtCKX3* (*CKX3*) or *ZmCKX1* (*CKX1*) nodulated by *M. loti* NZP2235 or infected with *M. incognita* race 1.
(a) Nodulated roots 2 weeks after inoculation. Note the lack of nodules in roots over-expressing *AtCKX3* or *ZmCKX1* compared to control.
(b) Hairy roots infected by *M. incognita* race 1, and 3 weeks after inoculation. Hairy roots overexpressing *CKX* genes have smaller gall number and size than the control hairy root.

Discussion

The cloning of genetically identified *Arabidopsis* cytokinin response genes has been an important step in understanding cytokinin function (Schmülling, 2002), and the isolation of cytokinin-responsive *ARR* genes, in particular, has facilitated investigation of the response of plant cells and tissues to cytokinins *in planta* (D'Agostino and Kieber, 1999). *ARR5* is a type A class member shown to be induced by cytokinins (Brandstatter and Kieber, 1998; D'Agostino *et al.*, 2000). We used this promoter fused to *GUS* and *GFP* reporters as an indicator of cytokinin responses during three important developmental processes: lateral root formation, nodulation, and RKN infection. We established that heterologous expression mediated by the *ARR5* promoter in *L. japonicus* recapitulates the native pattern obtained in *Arabidopsis*,

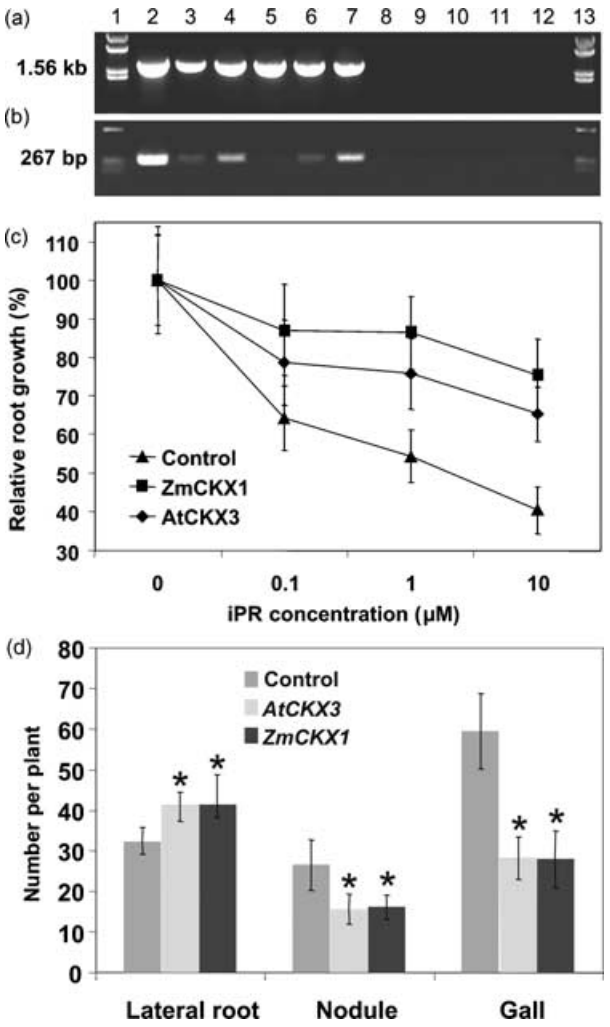

Figure 6. Modulation of cytokinin levels by transgenic expression of heterologous *CKX* genes.

(a) Amplification of a 1567-bp *AtCKX3* sequence from the genome of *Lotus* hairy roots. Lanes 1 and 13, λ *Bst*EII marker; lane 2, cloned *AtCKX3* template (positive control); lanes 3–7, independent hairy roots transgenic for *AtCKX3*; lanes 8–12, independent hairy roots transformed with vector alone (negative control).

(b) Amplification of a 267-bp *ZmCKX1* sequence from the genome of *Lotus* hairy roots. Lanes 1 and 13, λ *Bst*EII marker; lane 2, cloned *ZmCKX1* template (positive control); lanes 3–7, independent hairy roots transgenic for *ZmCKX1*; lanes 8–12, independent hairy roots transformed with vector alone (negative control).

(c) Root growth of transgenic hairy roots of *L. japonicus* to 5 days exogenous cytokinin iPR application, presented as per cent of root length without iPR application. Error bars represent 95% confidence intervals. $N = 45–84$.

(d) Lateral root, nodule and RKN gall number per plant in vector transformed control, and transgenic hairy roots of *L. japonicus* overexpressing *AtCKX3* or *ZmCKX1*. For lateral root number, hairy roots were grown on 1.2% agar slopes with half-strength B5 salts for 1 week after incubation on cefotaxime-containing medium, and lateral roots were counted per plant. $N = 9–23$. Nodule number per plant was counted in transgenic hairy roots of *L. japonicus* 2 weeks after inoculation with *M. loti* strain NZP2235 carrying *hemA::lacZ*. $N = 17–23$. For RKN gall number, hairy roots were inoculated with about 1000 *M. incognita* eggs, and were observed 3 weeks after inoculation. $N = 16–22$. Error bars represent 95.0% confidence intervals; bars marked with an asterisk are significantly different from the control, but not from each other.

The presence of transgenes was confirmed by PCR recovery from individual *L. japonicus* hairy roots (a,b).

where it reflects transcriptional responses to changes in cytokinin levels and/or sensitivity. It is important to note, however, that there may be tissue-specific affects on the *ARR5* promoter that may also contribute to the pattern of expression observed.

Cytokinins are synthesized in root tips and are transported to the shoot via the xylem (Davies, 1995), and the induction of *ARR5* expression we observed in the root tip and pericycle cells in vascular bundle is consistent with this known biology. However, not all pericycle cells along the length of the root express *ARR5*, suggesting that either some cells have a reduced sensitivity to cytokinins, or that the effective concentration of cytokinins somehow is lower in some parts of the root vascular bundle; the reason for and consequence of such regulation of the cytokinin response of vascular cells remain unclear.

It is generally assumed that cytokinins are essential for plant cell division, presumably through their influence on the cell cycle (Redig *et al.*, 1996; Zhang *et al.*, 1996), where they can act at the G_1/S-phase transition to initiate division in non-cycling cells (Francis and Sorrell, 2001). However, although cell division obviously is a pre-requisite for lateral root formation, we observed a striking repression of *ARR5* expression in the dividing cells of lateral root primordia in *L. japonicus*. It may be significant that most of the published results on the requirement of cytokinins in cell division come from experiments involving either shoot tissues or undifferentiated cell/callus cultures, or established meristems. Our results suggest that cytokinins may play different (or even opposite) roles during cell division in the mature root. However, it is also possible that this is a secondary consequence of altered auxin levels. We speculate that cytokinins have an inhibitory effect on initial cell division(s) during lateral root formation, and on overall root growth in plants. This explanation is supported by our *ARR5* expression data and increased lateral roots in *CKX* overexpressing *Lotus* hairy roots. This is consistent with observations that transgenic tobacco roots overexpressing *CKX* genes from *Arabidopsis* exhibit increased cell number and slightly reduced cell length because of an enhanced cycling of meristem cells (Werner *et al.*, 2001). Transgenic roots had increased number of lateral and adventitious roots, and an overall enhanced root growth. However, there was a decreased rate of new cell formation in cytokinin-resistant transgenic leaves of tobacco compared to wild-type leaves. Werner *et al.* (2001) attributed these conflicting effects of cytokinins on tobacco shoot and root growth to differential expression patterns of cytokinin targets in shoot and root meristems.

In contrast to lateral root formation, we observed a strong induction of *ARR5* expression in the curled/deformed root hairs and dividing nodule founder cells after inoculation of the root with *M. loti*, suggesting that cytokinins may play a role in the nodulation process in legumes. Several reports

have implicated a role for cytokinins in nodulation (Bauer *et al.*, 1996; Cooper and Long, 1994; Koltai *et al.*, 2001; Mathesius *et al.*, 2000; Minami *et al.*, 1996). Exogenous cytokinin application induced *ENOD40* expression in white clover root in a spatial and temporal pattern which mirrors that induced by Nod factor (Mathesius *et al.*, 2000). *ENOD40* is highly expressed in nodule primordia on *Medicago truncatula* (Koltai *et al.*, 2001) and is inducible by Nod factors (Minami *et al.*, 1996). Cooper and Long (1994) showed that non-nodulating Rhizobium mutants carrying a constitutive *trans*-zeatin secretion (*tzs*) gene from *Agrobacterium tumefaciens* stimulated the formation of nodule-like structures on alfalfa roots. Our observations of strong induction of *ARR5* expression in dividing cortical cells of the nodule primordium are consistent with a role for cytokinin. Our data showing a decreased nodule formation in *CKX* overexpressing *Lotus* hairy roots further support a positive role for cytokinins in nodulation. The lack of a cytokinin response at later stages of determinate nodule formation (i.e. in which the nodule meristem does not persist) in *L. japonicus* presumably reflects an absence of a meristem in the fully developed nodule and indicates a difference between lateral roots and determinate nodules.

Nutman (1948, 1949) reported that nodulated clover plants had fewer lateral roots than uninoculated plants, and proposed a physiological balance between lateral roots and nodules. As cytokinin has an opposite effect on lateral root formation and nodulation, this may represent one of the mechanisms by which the balance between lateral roots and nodules on a legume root is maintained.

In addition to any role for plant cytokinin in the RKN–host interaction, these parasites have been shown to be able to produce biologically active cytokinins *in vitro* (Bird and Loveys, 1980; de Meutter *et al.*, 2003), although whether the same is true *in planta* remains arcane. Our results indicate that RKN-infective larvae (L2) induce detectable *ARR5* expression neither at the site of root penetration nor during migration in the cortex. Once the L2 reach the differentiating vascular bundle, however, strong *ARR5* expression ensues. Whether this is indicative in a change in nematode secretions, or host sensitivity, is unknown. Although we did not establish the precise relationship between *ARR5* expression in the vasculature and the induction of GCs from parenchyma cells in the zone of root differentiation, it appears that the cytokinin response occurs before the L2 reach the differentiation zone. This is significant, as it suggests that a distinct host response occurs prior to the nematode committing to induce a feeding site (reviewed by Bird and Kaloshian, 2003), at which time the L2 cease to be motile. The primary events of feeding site formation are not understood at the cellular level, and our spatial mapping data neither support nor refute whether a cytokinin response occurs in those vascular parenchyma cells destined to become GCs. GCs are believed to be non-dividing, but do support karyokinesis and become highly multinucleate. Experiments with cell cycle inhibitors (de Almeida Engler *et al.*, 1999) revealed an initial transient requirement for cell cycle activation during GC formation; early application of hydorxyurea and oryzalin blocked GC formation, but application at later stages failed to impact nematode development, indicating normal GC function. Our results with *CKX* overexpressing *Lotus* hairy roots that had reduced gall formation indicated a requirement for cytokinins in the establishment of GC. As established GC did not exhibit *ARR5* expression, it must be the initial stages of GC induction that require cytokinins and consequently cell cycle activation (Goverse *et al.*, 2000). Action of the *Mi* locus to confer resistance to RKN also is temporally restricted to this period, and exogenous cytokinin application suppresses resistance (Dropkin *et al.*, 1969). Further, *Mi* is not effective against *M. hapla*, which produces smaller galls that typically sprout lateral roots (Lohar and Bird, 2003). It is interesting to note that *CKX* hairy roots formed smaller galls than control hairy roots in our experiments. Collectively, these results point to an interplay between a transient cytokinin influence in *Mi*-mediated resistance, which previously has been speculated by Bird (1996), Bird and Kaloshian (2003), and Bird and Koltai (2000).

The comparison of cytokinin responses during lateral root formation and symbiosis (nodulation and GC/gall formation) presented here makes it obvious that these two important post-embryonic plant organ developmental processes have different or even opposite cytokinin requirements. The cytokinin response is repressed for lateral root initiation, whereas it is induced for nodule and GC/gall initiation. Similarly, a cytokinin response was induced continuously in the root tip and in the lateral root meristem for the growth of the lateral root, whereas it was repressed in a mature non-growing determinate nodule and in already developed GCs. It appears therefore that the influence of cytokinins on cell division can be stimulatory or inhibitory. Plants presumably achieve this opposite effect using different pathways of cytokinin action, and possibly its effects are also modulated by other plant hormones, perhaps auxins. We are attempting to understand the other effectors.

Experimental procedures

Plant material and growth

Lotus japonicus ecotype Gifu B129 seeds were scarified and sterilized in concentrated sulfuric acid for 5 min, and then washed four to five times with sterile water. Seeds were germinated on wet filter papers in the dark for 2–3 days prior to hairy root production. Plants were maintained at 22°C for a 16-h day and 20°C for an 8-h night condition. *Lycopersicon esculentum* cv. Rutgers Large Red

seeds were surface-sterilized by sequential washes in 95% EtOH, 6% NaOCl, and four times in sterile water prior to germination on 0.8% agar containing 3% sucrose, half-strength MS salts, and full-strength Gamborg's vitamins. These and subsequent manipulations were performed in a laminar flow hood.

Expression constructs

We cloned a 1.6-kb fragment spanning the *ARR5* promoter gene into the binary vector, pBI101.1 (Clonetech, Palo Alto, CA, USA) as described by D'Agostino *et al.* (2000). A 1567-nt product corresponding to the *AtCKX3* transcript was amplified by reverse transcriptase (RT)-PCR (Bilyeu *et al.*, 2001) and cloned into pCR2.1TOPO vector (Invitrogen, Carlsbad, CA, USA), and subsequently subcloned into the *Bam*HI site of the pBI121 binary vector (Clonetech) between the *CaMV 35S* promoter and the *NOS* terminator. Similarly, cDNA corresponding to the *ZmCKX1* transcript (Morris *et al.*, 1999) was excised at *Bgl*II sites, and cloned into pBI121 *Bam*HI. Each binary construct was electroporated into *Agrobacterium rhizogenes* AR10 (Stiller *et al.*, 1997) using an Electro Cell Manipulator ECM 600 as per the manufacturer's recommendation (BTX Inc., San Diego, CA, USA).

Plant transformation and culture

Hairy roots were induced on *L. japonicus* seedlings grown on 1.2% agar slopes containing half-strength Gamborg's B5 basal salts and vitamins as described by Stiller *et al.* (1997). The composite plants (with transgenic hairy roots and wild-type shoots) were grown 5 days on 1.2% agar slopes with half-strength B5 basal salts and 300 $\mu g\ ml^{-1}$ cefotaxime (Gemini Bio-Products, Woodland, CA, USA) to eliminate live *Agrobacteria*, and were then transferred to 1.2% agar slopes with half-strength B&D medium (Broughton and Dilworth, 1971) for a further 5 days prior to inoculation.

Tomato cotyledons were excised 7 days after germination, repeatedly punctured with the scalpel tip, and were placed 2 days on filter paper overlaying 0.8% agar plates containing 3% glucose, full-strength Gamborg's vitamins, and MS salts, and 50 $\mu g\ ml^{-1}$ zeatin. Tomato cultures were maintained on a 16-h day/8-h night cycle at 26°C/24°C, respectively. Two milliliters of fresh, stationary-phase *A. tumefaciens* LBA4404 culture transformed with *ARR5::GFP* was inoculated into 8 ml of half-strength Luria–Bertani medium (LB) containing 42 mM NaCl, 6.7 mM glutamic acid, 27 mM mannitol, 1.5 mM K_2HPO_4, 400 μM $MgSO_4$, and 4 μM biotin, and were grown for 6 h. Cultures were pelleted, washed two times, and were re-suspended in 10 ml MS salts containing 3% sucrose and full-strength Gamborg's B5 vitamins. Each plate of explants was co-cultivated with 5 ml bacterial solution containing 10 mM acetosyringone, and was transferred to fresh 0.8% agar plates containing 5 μM zeatin for 2 days. Explants were then transferred every 14 days to fresh MS plates containing 2% sucrose, 10 μM zeatin, 500 μM inositol, and full-strength Nitsch vitamins. Shoots growing from callus were excised and rooted in MS salts containing 3% sucrose and full-strength Nitsch vitamins. Shoots and roots were transferred to potted soil for 1 week, and were then transferred to a greenhouse and maintained at 28°C until seeds were collected from ripe tomatoes.

Root sensitivity to exogenous cytokinins

Hairy roots were grown for 5 days on half-strength Gamborg's B5 medium agar slopes containing cefotaxime as described above, and were transferred to agar slopes containing half-strength B5 basal salts supplemented with either 0, 0.1, 1.0, or 10 μM iPR (Sigma, St Louis, MO, USA). Root tips were marked at the time of transfer, and 45–84 roots were measured for growth (length) 5 days later. The growth for each treatment was averaged and analyzed for statistical significance. One group of plants was grown on 1.2% agar slopes with half-strength B5 salts for further 1 week after incubation on cefotaxime-containing medium, and lateral roots were counted per plant (9–23 plants per treatment) to observe the effect of transgenes on root growth.

Nodulation experiments

Nitrogen-starved composite plants grown on agar slopes were transferred to vermiculite and watered with half-strength B&D solution. One week after transfer, plants were inoculated with *M. loti* strain NZP2235 marked with a *hemA::lacZ* construct (Schauser *et al.*, 1998). Roots were stained with X-gal 14 days after inoculation as described by Boivin *et al.* (1990), and the nodules were counted visually. A minimum of 17 plants was used for each treatment, and the nodule number for each treatment was statistically analyzed.

Root-knot nematode infection

Populations of *M. incognita* and *M. hapla* were maintained in the greenhouse on *L. esculentum* cv. Rutgers Large Red, and their identity was periodically confirmed by isozyme analysis (Esbenshade and Triantaphyllou, 1990). Nematode eggs were bulk extracted with 0.5% NaOCl (Hussey and Barker, 1973) and stored for up to a week at 4°C. Composite *L. japonicus* plantlets were potted under conditions optimized for nematode infection (Lohar and Bird, 2003), and each of them were inoculated with approximately 1000 nematode eggs. Plants were uprooted beginning from 1 week after inoculation for histochemical examination. Typically, 50 independent hairy roots were observed for GUS staining for each infection. Similarly, hairy roots transgenic for *AtCKX3*, *ZmCKX1*, and vector control were observed 3 weeks after inoculation, and gall number was counted for each plant. At least 16 plants were used for each type of transgenic root.

In situ *hybridization*

Plant growth pouches (MegaInternational, St Paul, MN, USA) were each sown with three surface-sterilized seeds and 2-week-old seedlings were inoculated for 12 h with 10 000 *M. incognita* L2. Roots and galls were excised at appropriate time periods post-infection, and were either fixed according to Koltai and Bird (2000) or observed under UV illumination. Fixed galls were prepared, sectioned, and subjected to *in situ* hybridization following Koltai and Bird (2000). RT was performed using primer 5′-TGTTGACGAGGGTGTCTC-3′. PCR was performed using the same RT primer and 5′-ATATGAAGCGGCACGACT-3′.

Histochemical GUS assay

Roots were incubated overnight at 37°C in 0.05 M sodium phosphate, 1.0 mM potassium ferricyanate, 1.0 mM potassium ferrocyanate, 1 mM EDTA, 1 $\mu l\ ml^{-1}$ Triton X-100, and 102.5 $\mu g\ ml^{-1}$ β-glucuronic acid. Stained roots were mounted on microscope slides and photographed either using a compound microscope (Optiophot, Nikon, Tokyo) equipped with Nomarski

differential interference contrast (DIC) optics, or a stereomicroscope. At least 25 plants were observed for *ARR5* expression analysis during lateral root formation, and after inoculation of roots with either *M. loti* NZP2235, *M. loti NodC* mutant, or mock inoculation. Nodules and galls were also hand-sectioned, stained with β-glucuronic acid, and mounted on slides for observation and photography. For light microscopy, nodule and gall samples were fixed, dehydrated, infiltrated with paraffin, and 25-μm sections were obtained using a rotary microtome (Reichert-Jung, Austria) as described by Graham and Joshi (1995). Sections were counterstained with Safranin O.

Genomic DNA isolation and PCR

Genomic DNA was isolated from hairy roots using a DNeasy Plant Minikit as described by the manufacturer (Qiagen Inc., Valencia, CA, USA). The transgenes were amplified from approximately 100 ng hairy root genomic DNA in a 50 μl PCR reaction mix containing 0.2 μM each of the following forward and reverse primers: *AtCKX3*: 5′-ATGGCGAGTTATAATCTTCG-3′ and 5′-CTAACTCGAGTTTATTTTTTG-3′; *ZmCKX1*: 5′-AACAAATCCATGTGGGACG-3′ and 5′-TGGGGTCGTACTTGTTCTTC-3′.

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